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Vitis sp. response to *Xylella fastidiosa* strain CoDiRO

EFSA Panel on Plant Health (PLH)

Abstract

Following a request from the European Commission, the EFSA Panel on Plant Health assessed a scientific report submitted by the Italian Authorities to the European Commission to support a request to delist *Vitis* sp. from Annex I ('specified plants') of the Commission Implementing Decision (EU) 2015/789 of 18 May 2015 to prevent the introduction into and the spread within the Union of *Xylella fastidiosa* (Wells et al.). The report comprised (i) surveys to detect *X. fastidiosa* in vineyards located in the epidemic zone of CoDiRO with high numbers of diseased olive trees; (ii) inoculation experiments to infect grapevine with a *X. fastidiosa* isolate 'De Donno' from CoDiRO diseased olives; and (iii) vector transmission experiments with *X. fastidiosa* infective *Philaenus spumarius*. The Panel acknowledges the difficulties in providing evidence about this hitherto unknown pathogen/vector/host interaction to support the hypothesis that a plant species cannot be infected with a pathogen. Although field surveys to detect *X. fastidiosa* in grapevine were negative, there was no supporting information on infective vector populations present in the vineyards. Hence absence of infection pressure cannot be excluded. Furthermore the failure to infect grapevine plants either by artificial inoculation or by vector transmission might be due to inoculation conditions not appropriate to induce infections in grapevine. The detection of *X. fastidiosa* DNA in inoculated grapevine plants even 12 months after inoculation, although localised at the inoculation points, cannot exclude that the DNA amplified by qPCR was from viable cells. The results presented are coherent and provide converging lines of evidence that grapevine (*Vitis vinifera*) is not a major susceptible host of *X. fastidiosa* strain CoDiRO. However, from the experimental evidence it is premature to exclude that systemic infections of *V. vinifera* and *Vitis* sp. occur and that infections at limited foci could serve as a source of inoculum.

Keywords: detection, grapevine, inoculation, pathogenicity assay, *Philaenus spumarius*, subspecies *pauca*, vector transmission, vineyard survey

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Amendment: An editorial clarification was carried out on p.8 which does not materially affect the content of the opinion. The following text: 'such as *Scaphoideus titanus* Ball (Hemiptera: Cicadellidae) which has already been observed in vineyards in Apulia (Digiaro et al., 2014)' was removed (along with the citation from the reference list) as the Panel considered it could be misinterpreted.

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1. Introduction

1.1. Background and Terms of Reference as provided by the requestor[1]

The purpose of this mandate was a request, pursuant to Article 29 of Regulation (EC) No 178/2002[2], to provide 'scientific advice in the field of plant health as regards the regulated harmful organism *Xylella fastidiosa* (Wells et al.).

In particular, given the uncertainty of the complete host range of *Xylella fastidiosa* strain CoDiRO, as well as the pathogenicity tests which were still ongoing during the review process of the EU emergency measures, the Commission and Member States agreed at the time to include *Vitis* sp. in the list of specified plants (Annex I) of Commission Implementing Decision 789/2015/EU[3], while waiting for final confirmatory results.

In the meantime, the Italian Authorities have submitted to the Commission the results of pathogenicity tests and analyses carried out to verify the susceptibility of *Vitis* sp. to *Xylella fastidiosa* strain CoDiRO, and, given the results, requested its delisting from the EU Decision.

Consequently, in order for the Commission and Member States to further analyse such information and make a decision in the relevant Standing Committee, EFSA has been asked to provide scientific advice on the information submitted by the Italian Authorities'.

1.2. Interpretation of the Terms of Reference

The Terms of Reference (ToR) focus on the results provided in the scientific report 'Pathogenicity tests and analysis to verify the susceptibility of grapevines to *X. fastidiosa* strain CoDiRO' dated 2 September 2015 and prepared by the National Research Council UOS of Bari and the University of Bari Aldo Moro (hereafter referred to as 'the report').

The report is composed of three parts:

(i) surveys in vineyards and nurseries located in the epidemic zone of diseased olive trees with CoDiRO symptoms (acronym of 'Complesso del Disseccamento Rapido dell'Olivo' equivalent to the English 'olive quick decline syndrome', OQDS);

(ii) inoculation experiments to infect grapevine (*Vitis vinifera* L.) with a *X. fastidiosa* isolate 'De Donno' from CoDiRO diseased olives; and

(iii) transmission experiments with CoDiRO strain infective *Philaenus spumarius* L. (Hemiptera: Aphrophoridae) adults to infect *V. vinifera* plants. Considering the potential asymptomatic presence of *X. fastidiosa* observed in many plant species and the risks connected to its movement in the EU via asymptomatic plants, the Panel considered in its evaluation both the susceptibility of *Vitis* sp. to disease caused by *X. fastidiosa* representing strain CoDiRO and the potential of *Vitis* sp. to carry the bacterium without expressing symptoms.

The EFSA Panel on Plant Health (PLH) wishes to emphasise that the colloquial use of the term *X. fastidiosa* strain CoDiRO is erroneous. The assignment of *X. fastidiosa* subsp. *pauca* isolates associated with CoDiRO in olives to a single CoDiRO strain is not warranted. Hence the term 'strain CoDiRO' is used in this document for reasons of coherence with the terminology applied in the mandate, the research report and in literature (Giampetruzzi et al., 2015).

The Panel also points out that the evidence on *Vitis* sp. and its *Xylella* pathogens available in scientific literature is largely from experiments conducted on *V. vinifera* and rarely includes other *Vitis* sp. Therefore, the Panel's assessment exclusively refers to the grapevine species *V. vinifera* as a potential host of *X. fastidiosa* strain CoDiRO and cannot extend its assessment to other *Vitis* species.

[1] Submitted by European Commission, ref. SANTE.E2/PdR/pm (2015) 4131582

[2] Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. OJ L 31, 1.2.2002, p. 1–24

[3] Commission Implementing Decision (EU) 2015/789 of 18 May 2015 as regards measures to prevent the introduction into and the spread within the Union of *Xylella fastidiosa* (Wells et al.). OJ L 125, 21.5.2015, p. 36–53

The assessment is thus based on the methods used, results obtained and conclusions made to substantiate that *V. vinifera* is not a host for the strain CoDiRO. The report by itself was not considered to provide sufficient information to support its conclusions. Therefore, the Panel requested the authors to provide further clarifications on pathogenicity tests, transmission trials and surveys conducted prior to September 2015. An interview with researchers responsible for the report during a web conference with members of the working group (WG), held on 4 of November 2015, provided the necessary clarifications for this scientific opinion.

For the assessment of the report, the Panel focused on the results of surveys and trials concluded and did not take into consideration preliminary information from ongoing studies for which results were still pending (e.g. presence and population dynamics of the vectors including *P. spumarius* in vineyards or studies on other host species).

2. Data and Methodologies

2.1. Data

To evaluate the report at the beginning of the mandate, an extensive literature search on the association of all currently identified *X. fastidiosa* subspecies and strains with *Vitis* sp. was conducted. Keywords used were '*Xylella fastidiosa*', '*Vitis*', 'inoculation', 'vector' and variants as search terms. Searches were carried out on the research platform ISI Web of Science. The references retrieved were reviewed together with those cited in the report provided with the mandate and references cited in the relevant sections of the EFSA risk assessment on *Xylella* produced earlier (EFSA PLH Panel, 2015). Further references and information were obtained from citations within the reviewed references and from experts.

2.2. Methodologies

The assessment was conducted in line with the principles described in the EFSA Guidance on transparency in the scientific aspects of risk assessment (EFSA Scientific Committee, 2009). The present document is structured according to the Guidance on the structure and content of EFSA's scientific opinions and statements (EFSA Scientific Committee, 2014). For a thorough evaluation of *Vitis* sp. as a possible host of strain CoDiRO, the Panel considered all literature relevant to support a scientific opinion on an unprecedented situation (unknown pathogen, invasion into a new geography, new hosts, new insect vectors). The discussion is divided in three sections reflecting the structure of the report: (i) surveys in vineyards in the CoDiRO epidemic zone; (ii) pathogenicity tests; (iii) vector transmission experiments. Uncertainties are identified and discussed with regard to their impact on the final conclusions.

3. Assessment

3.1. Introduction

The bacterium *X. fastidiosa* is found in many plant species; in some, it exists as an endophyte in the xylem vessels of its host which can then appear unaffected by its invasion (e.g. Hopkins, 1989) while in other species, including the important crops almond, citrus, coffee, grapevine, and olive, it can be a serious pathogen, causing a wilting or scorching disease (EFSA PLH Panel, 2015). It is not known what characteristics determine endophytic vs. pathogenic growth but environmental factors may play a role (Choi et al., 2013).

Xylella fastidiosa is a distinct species of which at least four genetically distinct subspecies have been identified. In general, each *X. fastidiosa* subspecies is considered to be associated with a specific list of hosts: subsp. *fastidiosa* with grape and almond, *multiplex* with a large number of fruit and forest tree species, *pauca* with citrus and coffee in South America, and *sandyi* with oleander (EFSA PLH Panel, 2015). However, in addition to a plant species being a major host for a particular *X. fastidiosa* subspecies, the same plant can host more than one *X. fastidiosa* subspecies (Table 1).

Within the *X. fastidiosa* subspecies, bacterial isolates that are associated with particular crop diseases have been characterised. For *X. fastidiosa* subsp. *pauca*, isolates colonizing citrus, causing citrus variegated chlorosis disease (CVC), and isolates from coffee causing coffee leaf scorch disease (CLS)

were described. Although sympatric in their occurrence, these distinct isolates were associated with one host only and not with the other, indicating biological isolation between CVC isolates and isolates causing CLS in coffee. Consequently *X. fastidiosa* subsp. *pauca* isolates causing citrus variegated chlorosis (CVC) and those causing coffee leaf scorch (CLS) were considered as distinct strains (Almeida et al., 2008).

Xylella fastidiosa isolates associated with the severe CODiRO disease of olive in Apulia also belong to subsp. *pauca*. However, by applying multi locus sequence typing (MLST), a technique used to identify, discriminate and cluster *X. fastidiosa*, these isolates were all assigned to a sequence type, ST53 (Elbeaino et al., 2014a; EFSA PLH Panel, 2015). Also isolates from coffee and oleander in Costa Rica were assigned to ST53 (Nunney et al., 2014a), that were phylogenetically separated from the citrus CVC and the coffee CLS strains from South America.

A *X. fastidiosa* isolate transmitted from a CoDiRO diseased olive in Apulia to periwinkle has been well characterized and a draft genome sequence of the CoDiRO strain has been assembled (Giampetruzzi et al., 2015). Results surveys in natural environments show that it is capable of infecting a very broad range of hosts under natural conditions; a provisional list already includes almond (*Prunus dulcis*), cherry (*Prunus avium*), coastal rosemary (*Westringia fruticosa*), genista (*Spartium junceum*), golden wattle (*Acacia saligna*), Mediterranean buckthorn (*Rhamnus alaternus*), myrtle (*Myrtus communis*), oleander (*Nerium oleander*), periwinkle (*Vinca minor* and *Catharanthus roseus*), rosemary (*Rosmarinus officinalis*), and September bush (*Polygala myrtifolia*) (Saponari et al., 2013 and 2014a; Elbeaino et al., 2014a; Digiaro and Valentini, 2015). More recently, CoDiRO strain has been found in *Asparagus acutifolius*, *Cistus creticus*, *Dodonaea viscosa purpurea*, *Euphorbia terracina*, *Grevillea juniperina*, *Laurus nobilis*, *Lavandula angustifolia*, *Myoporum insulare*, *Westringia glabra* (information received during the hearing with the authors of the report). While still provisional, this list covers a range of important agricultural and ornamental plant species belonging to different botanical families. Although *X. fastidiosa* isolates from periwinkle (Giampetruzzi et al., 2015) and from olive belong to the same ST53 genotype (Elbeaino et al., 2014a), it is unclear at this moment if this also extends to *X. fastidiosa* found in other hosts in the Apulia region. Previous research has shown that *X. fastidiosa* isolates differ in virulence and host specificity even when found in the same area (Almeida and Purcell, 2003; Oliver et al., 2014, 2015).

It has been speculated that the presence of *X. fastidiosa* strain CoDiRO in Apulia is the result of a recent introduction (Frisullo et al., 2014). This same strain has been found on plant species from unrelated taxons such as olives (order Laminales) and oleander (order Gentianales), indicating that the extent of host adaptation cannot be predicted (Almeida and Nunney, 2015; EFSA PLH Panel, 2015). The emergence of new diseases caused by *X. fastidiosa* may be the result of an invasion of the bacterium into new geographic areas. Recombination (genetic exchange between strains) (Nunney et al., 2014b; Almeida and Nunney, 2015) and changes in gene regulation (Killiny and Almeida, 2011) have also been shown to broaden the host range of *X. fastidiosa*. In nature, Pierce's disease symptoms on grapevine are only caused by *X. fastidiosa* subsp. *fastidiosa*. There is only one report of artificial inoculation of isolates of *X. fastidiosa* subsp. *pauca* from citrus and coffee to grapevine resulting in scorching symptoms (Li et al., 2002). However, because of the aggressive inoculation procedure used (very high inoculum and large number of punctures), it is unclear whether the observed symptoms reflect actual infections or rather are the consequences of the method used.

Table 1: Records of *Xylella fastidiosa* subspecies found on the same host species. For a full review of all potential hosts currently found infected by *X. fastidiosa*, the reader should refer to EFSA PLH Panel, 2015.

Plant species	*Xylella fastidiosa* subspecies	Symptoms	Infection type	References
Almond *Prunus dulcis*	*fastidiosa*	Leaf scorch	natural infection, experimental inoculation	Chen et al., 2005; Hernandez-Martinez et al., 2006; Nunney et al., 2013; Krugner et al., 2014
	multiplex	Leaf scorch	natural infection, experimental inoculation	Chen et al., 2005; Hernandez-Martinez et al., 2006; Nunney et al., 2013; Krugner et al., 2014
	pauca strain CoDiRO	Leaf scorch	natural infection	Digiaro and Valentini, 2015; Elbeaino et al., 2014a
Cherry *Prunus avium*	*fastidiosa*		natural infection	Hernandez-Martinez et al., 2007
	pauca strain CoDiRO	Scanty vegetation and bud failure but not leaf scorch	natural infection	Saponari et al., 2014a
Grapevine *Vitis* sp. and *Ampelopsis cordata*	*fastidiosa*	Pierce's disease	natural infection, experimental inoculation	Hernandez-Martinez et al., 2006, 2007; Nunney et al., 2013; Elbeaino et al., 2014a
	multiplex	Present in *Ampelopsis cordata* (peppervine)	natural infection	Morano et al., 2008
	pauca	Pierce's disease	experimental inoculation	Li et al., 2002
Myrtle-leaf milkwort *Polygala myrtifolia*	*multiplex*	Drying of the foliage	natural infection	EPPO, 2015
	pauca strain CoDiRO	Apical leaf necrosis and branches drying	natural infection	Saponari et al., 2014a
Oleander *Nerium oleander*	*pauca* strain CoDiRO	Leaf scorch	natural infection	Saponari et al., 2014a; Cariddi et al., 2014
	pauca ST53		natural infection	Nunney et al., 2014a
	sandyi	Leaf scorch	natural infection, experimental inoculation	Almeida et al., 2008; Hernandez-Martinez et al., 2006, 2007; Nunney et al., 2013
Olive *Olea europaea*	*multiplex*	Leaf scorch and branch die back, not well correlated with Xf infection	natural infection, experimental inoculation	Krugner et al., 2014; Nunney et al., 2013
	pauca (Argentina)	Desiccated leaves and leaf scorch	natural infection	Haelterman et al., 2015
	pauca strain CoDiRO	Olive quick decline syndrome	natural infection	Saponari et al., 2014a; Elbeaino et al., 2014a
Blueberry *Vaccinium* sp.	*multiplex*	Leaf scorch, dieback and stem yellowing	natural infection, experimental inoculation	Oliver et al., 2015; Hopkins et al., 2012; Oliver et al., 2014; Parker et al., 2012
	fastidiosa	Leaf discoloration, scorch and leaf drop	experimental inoculation	Oliver et al., 2015; Hopkins et al., 2012

3.2. Surveys in vineyards and nurseries in the CoDiRO epidemic zone

Pathogen surveys provide data on the incidence of pathogens over a geographical range or in particular hosts (FAO, 2011). Surveys often consist of visual inspection for disease symptoms and estimation of symptom severity scores. Such surveys provide information on the distribution and prevalence of the pathogen as well as the susceptibility of the host(s) (Tubajika et al., 2004; Park et al., 2011). Pest surveys are supported by appropriate diagnostic services (FAO, 2011) , enabling the

accurate detection and identification of pathogens even in the absence of symptoms (Hernandez-Martinez et al., 2007; Cariddi et al., 2014; Elbeaino et al., 2014a; Loconsole et al., 2014). Verification of diagnosis is especially necessary for pathogens like *X. fastidiosa*, causing mild and/or non-specific symptoms (such as wilting or scorching) (Krugner et al., 2014).

Surveys for the presence of disease potentially caused by *X. fastidiosa* CoDiRO in grapevine were conducted in vineyards during November 2013, late summer 2014, January 2015, and September 2015. In addition, samples were taken from certified mother plants and rooted vines in grapevine nurseries of the Otranto area. As indicated in the report, surveyed vineyards and nurseries were in the CoDiRO epidemic zone. No specific symptoms of Pierce's disease were spotted during the surveys. The sampling strategy used was not described in detail but was clarified through information provided by the authors during the hearing (see section 1.2.). It appears that samples were collected across each vineyard from approximately 10 randomly selected vines. Samples collected were tested for the presence of *X. fastidiosa* by both ELISA and PCR during the first three surveys while during the last one (September 2015) only plants with ambiguous results by ELISA were also tested by PCR (three out of 243 ELISA tested samples) (Annex 1 – Results of the field survey conducted on 1–9 September 2015). All results from the surveys in vineyards and nurseries shown in the report indicate that the CoDiRO strain has not been detected in the sampled grapevines. Although a much higher sample size would be required to confirm absence of CoDiRO strain from the surveyed *Vitis* sp., considering the overall disease pressure in the epidemic zone and the severity of the disease in olive, the statement on the health status of grapevine is conceivable.

The Panel identified several concerns regarding the detection of *X. fastidiosa* CoDiRO in grapevine.

1. Compared with olive, that supports high multiplication rates, systemic movement, and symptom development of CoDiRO strain, grapevine susceptibility can be assumed to be lower. However, it is possible that low level infections can exist with no or limited symptomatology (Almeida and Purcell, 2003). A lower limit of CoDiRO detection by ELISA and PCR was determined by spiking plant homogenates with inactivated bacterial cultures and a detection limit of 10^4 CFU/ml was determined for both assays. ELISA tests are reliable for detection of the CoDiRO strain in olives and other susceptible hosts with high concentrations of bacteria (Loconsole et al., 2014). However it is not clear whether ELISA would detect low concentrations of bacterial cells (Costa et al., 2004). And given that it has a similar sensitivity, it is not clear either that PCR can detect infections at low concentrations.
2. Similarly, the surveys were conducted during different seasons. Symptoms of Pierce's disease in grapevine are most severe in late summer (Hopkins, 1981; Feil et al., 2003; Costa et al., 2004). Surveys done at other times of the year are likely to be less reliable in detecting bacterial infections. Therefore the Panel considered the survey data from September 2015 to be probably the most reliable.
3. The link between the CoDiRO strain and its host plant is provided by the insect vector (Almeida and Nunney, 2015; EFSA PLH Panel, 2015). Even high inoculum concentrations in an epidemic zone containing many severely infected host plants (olives) could only be expected to result in transmission to grapevine if the vectors visit and feed on the plant (Daugherty et al., 2009; Daugherty and Almeida, 2009). However, the surveys did not include information about *P. spumarius* presence and density in the sampled vineyards (Elbeaino et al., 2014b; Saponari et al., 2014b) or data on other putative vector species. The size of the vector population found in the field, their number found on grapevine plants, and the number carrying CoDiRO by PCR would provide an accurate assessment of the inoculum pressure in vineyards. Although *P. spumarius* has been observed on *Vitis* sp. (Carle and Moutous, 1966; Aldini et al., 1998; Braccini and Pavan, 2000; Pavan, 2006; Daane et al., 2010; Kunz et al., 2010; Avramov et al., 2011), because of the missing insect vector data, it is not possible to confirm that the sampled grapevine plants had been exposed to high inoculum pressure.

3.3. Pathogenicity tests

Biological assays provide the most conclusive evidence that pathogen and plant undergo compatible interactions; successful colonisation of the host consists of both multiplication of the pathogen and its systemic movement through the plant (Newman et al., 2003; Krivanek and Walker, 2005; Fritschi et al., 2008; Baccari and Lindow, 2011). The identification and consistent detection of a pathogen in a particular plant makes disease association possible but, because of the complex origin of diseases, only the fulfilment of Koch's postulates provides the ultimate proof. However, Koch's postulates are sometimes impossible to fulfil and for many plant diseases, including some caused by *X. fastidiosa,* this final evidence and confirmation of disease aetiology is pending (Huang et al., 2003; Randall et al., 2009). In at least two cases, *X. fastidiosa* has been isolated from diseased olive plants, yet back transmission assays to infect the original host failed (Krugner et al., 2010, 2014; Saponari et al., 2014b). In a recent experiment published by Krugner et al. (2014), *X. fastidiosa* subsp. *Multiplex* was isolated from symptomatic olives in California and mechanically inoculated to almond, grapevine and also back to olive. In almonds, symptoms were observed. In grapevine neither symptoms were observed nor was the pathogen detected by PCR. In olives, *X. fastidiosa* was only detected for a few months after inoculation and during the time of observation infections remained asymptomatic. The authors were unable to confirm the bacterium as the causal agent of olive leaf scorch disease (Krugner et al., 2010, 2014).

The Panel acknowledges that experiments to prove the host status of a plant for a specific pathogen are among the most challenging in plant pathology. As observed for *X. fastidiosa* and for other pathogens, the introduction of a bacterium into a plant and the observation of its systemic movement within the host do not always result in persistent infections (Purcell and Saunders, 1999; Feil et al., 2003; de Souza Prado et al., 2008). In some cases, bacterial populations can decline even after an extended period of dispersion/invasion in the plant (de Souza Prado et al., 2008). These transient disease situations complicate the assessment of the susceptibility of a host because even hosts that support only small and transient bacterial populations might still function as sources of inoculum for insect vectors (Hill and Purcell, 1997; Purcell and Saunders, 1999; Wistrom and Purcell, 2005; Marucci et al., 2005; Krugner et al., 2014).

Experiments to inoculate plants by (mechanical) introduction of bacteria and their translocation to the xylem are intended to mimic the action of vectors that are competent to deliver the pathogen to the plant (Wistrom and Purcell, 2005; Backus et al., 2015). Such experimental conditions are, however, highly artificial and, particularly when unknown interactions are to be studied, each variable of the procedure (e.g. bacterial cell density, pricking sites and intensity) has to be optimized. The experiments described in the report to infect grapevine with strain CoDiRO use the standard mechanical inoculation procedure. The bacterial suspension was introduced into the xylem by placing a cell suspension at three sites (basal nodes) on the stem and piercing several times through the droplet to reach the xylem for absorption of the cell suspension. The inoculation experiment was repeated four times on *V. vinifera* plants belonging to the cultivar Cabernet Sauvignon (a cultivar successfully infected experimentally in the US by Li et al. (2002) with the CVC strain of *X. fastidiosa pauca*); the first on three plants and the next three on 10 plants each. Parallel inoculation experiments were also conducted on olive and periwinkle as known susceptible hosts to confirm the efficacy of the procedure. Experiments on olive plants were performed three times with 6, 10 and 20 plants respectively; details of the experimental design for periwinkle were not provided.

This report provides the first evidence of a successful back transmission of strain CoDiRO to infect olives. Using the inoculation method described, olive seedlings became infected with strain CoDiRO and tested positive by qPCR. Bacterial DNA was detected in upper parts of the inoculated plants and in the roots, indicating basipetal movement of bacteria. Bacteria were also isolated from stem sections and cultured on media. Although the exact number of plants infected / plants treated was difficult to extrapolate from the tables in the report, the infectivity of the *X. fastidiosa* isolate used was proven and to olives readily became infected. However, except for 'the appearance of inconsistent symptoms of leaf scorching' (page 10 of the report) most inoculated plants remained asymptomatic and the CoDiRO symptoms were not confirmed. This missing link – bacterial infection and induction of symptoms – does not exclude future symptom development and thus it remains uncertain whether symptom progression into CoDiRO would occur even after the one year observation period. The

successful infection of olives with strain CoDiRO, however, provides evidence for a system competent to infect olives.

Notwithstanding, it is not understood why olive was considered as a sufficient reference and infection control for the grape experiments. Each pathogen-host combination presents a unique challenge and thus an additional useful control would be the inoculation with *X. fastidiosa* subsp. *fastidiosa* isolates that are known to infect grapevine. However it is also understood that regulatory restrictions may severely limit the ability to conduct such tests. Moreover, a single *V. vinifera* cultivar (Cabernet Sauvignon) was used for those experiments, without taking into consideration the potential host response variability across different grapevine genotypes (Raju and Goheen, 1981; Krivanek and Walker, 2005; Baccari and Lindow, 2011; Wallis et al., 2013). Therefore, additional control inoculations and the inclusion of more than a single grapevine cultivar and scion/rootstock combination would have been useful to more strongly support the broad scope of the report.

The Panel identified several elements of concern regarding these pathogenicity tests.

1. Assuming that the sensitivity of grapevine to infection with strain CoDiRO is low (unlike *X. fastidiosa* subsp. *fastidiosa* causing Pierce's disease in grapevine) inoculating grapevine with strain CoDiRO can be inefficient. Thus, increasing the inoculum pressure by means of higher numbers of inoculation points and/or re-infection over time could have been considered in order to induce infection. Li et al. (2002) described the mechanical inoculation and subsequent infection of grapevine with coffee and citrus isolates of *X. fastidiosa* subsp. *pauca* leading to visible scorching symptoms as early as one month after inoculation. In contrast to the pin-pricking method used in the trials reported, grapevine plants were injected at 20 points with 10^8 to 10^9 CFU of bacteria per ml suspension using a syringe and a 20G needle. This treatment with high volumes of concentrated bacterial suspension has arguably considerable side effects making it difficult to discriminate between stress induced by the treatment and disease caused by the pathogen (de Souza Prado et al., 2008). Despite the fact that lower inoculum concentrations are recommended to avoid non-host reactions (Schaad et al., 2004), higher concentrations of inoculum are required in case of cross-inoculation experiments with heterologous strains, as in the current trial, and as observed by de Souza Prado et al. (2008) where isolates of *X. fastidiosa* from citrus and coffee were reciprocally inoculated. A more comprehensive method to infect olives was presented by Krugner et al. (2014) who infected 1 year old olive plants by inoculation of cell suspensions of approximately 10^8 cells/ml at three locations of the stem (similar to the method presented in the report), but repeated the treatment four times over a six-month period (March to September). Despite this very comprehensive treatment regime to back transmit a pathogen to its original host, only a few plants became infected, indicating the difficulty of such experiments. By analogy, it can be argued that the low number of grapevine plants used for the experiments (in particular the first one, started on 31 July 2014, which involved three plants only) and that the method of inoculation used could have had limitations in their efficiency and, therefore, in their ability to detect a low transmission rate to grapevine. As reported in Tables 3–6, bacterial DNA was found near the grapevines inoculation sites only and was detectable up to 12 months after inoculation (Table 3) and, more commonly, towards the later months of sampling in the experiments A, B, and C (Tables 3–5). There was no detectable DNA in petioles and stems above the inoculation points. Related to this last issue, data regarding CoDiRO strain populations in plants are expressed in Cq values, which are valuable to compare relative amounts under the same laboratory conditions, but are not useful for understanding the actual population size of CoDiRO strain. Population density, however, is an important piece of information for assessing endophytic vs. pathogenic growth. Using a standard curve in parallel with the qPCR reactions would have been useful to relate Cq values determined by qPCR to bacterial cell concentrations (CFU/g), allowing comparisons with research conducted by others (for instance research showed that populations of 10^7-10^9 CFU/g are needed for systemic movement, as in Hill and Purcell, 1995).

2. These results, together with the inability to isolate and culture bacterial colonies from previously inoculated grapevine plants suggest that the qPCR amplification may correspond to dead cells, as suggested in the report. Another possibility is that the detected DNA corresponds to viable but non-culturable (VBNC) cells, a state that has not been proven yet for *X. fastidiosa* inside the host, but observed *in vitro* (Navarrete and de la Fuente, 2014). To

understand if what the researchers were detecting was living, dead or VBNC cells, approaches such as dilution plating, or a qPCR technique allowing differentiation of viable cells (such as qPCR-EMA or -PMA) would have been useful. Moreover, electron microscopy would have helped to visualize bacteria colonization of the xylem vessels and to determine if they are forming biofilms – a strategy for survival used by *X. fastidiosa* and other xylem pathogens. The understanding of whether the DNA detected refers to live cells in the inoculated grapevines however is fundamental to conclude if this host can serve as an asymptomatic reservoir for strain CoDiRO or not. From the data provided it is hard to interpret how bacterial DNA persisted for several months without degradation or removal by xylem flow, if bacterial cells had died. This question is important since previous research has demonstrated that *X. fastidiosa* can multiply in some hosts without systemic movement (Hill and Purcell, 1995) and insect vectors can acquire bacteria from non-systemic hosts (Purcell and Saunders, 1999). Therefore, if CoDiRO bacterial cells were still alive inside the inoculated grapevines, they could potentially be transmitted by insects feeding on the xylem sap of grapes (including *P. spumarius*).

In general, the Panel acknowledges that experiments providing negative evidence inherently present many difficulties. In this case, considering the limited number of grapevines tested, the questions raised concerning the stringency of the inoculation procedure, the use of a single grapevine variety and the short time of observation for some repetitions of the assay, the Panel is unable to provide an unequivocal statement on the susceptibility of *V. vinifera*, and even less of *Vitis* sp., to infection by strain CoDiRO.

3.4. Vector transmission experiments

In nature, *X. fastidiosa* dissemination and spread relies mainly on transmission by xylem sap sucking vectors, mostly sharpshooters and spittlebugs (Hemiptera: Clypeorrhyncha). Critical to insect vector transmission of bacteria is the acquisition of the bacteria from a source plant, retention of the bacteria in the insect foregut, inoculation or release of bacteria to a new host, and subsequent plant infection (Almeida et al., 2005; Almeida and Purcell, 2006; Chatterjee et al., 2008; Daugherty and Almeida, 2009; Daugherty et al., 2009; Backus and Morgan, 2011; Killiny and Almeida, 2014). Acquisition depends on bacterial population density and varies with host plant and vector species (Hill and Purcell, 1997; Lopes et al., 2009). The feeding behaviour of the vector is crucial for the inoculation of bacterial cells to plants (Almeida and Nunney, 2015) and vector ecology drives the epidemiology of disease (Redack et al., 2004; Almeida et al., 2005). In nature, insects are more efficient vectors to infect plants than artificial inoculation methods (Wistrom and Purcell, 2005), provided that the conditions for insect, plant and bacteria are optimal for all phases involved in pathogen transmission. Vector transmission experiments under laboratory conditions ignore ecological characteristics, habitat selection, host plant preference, etc. but rather seek to clarify whether a particular insect species is capable to vector a given pathogen to a given host plant. For strain CoDiRO indirect evidence for *P. spumarius* being a vector species was provided by the detection of bacteria in a large number of specimens collected in the epidemic zone. Its vector status was confirmed by transmitting strain CoDiRO to periwinkle (*Catharanthus roseus*), a susceptible herbaceous plant which has been classically used as control host for infection trials with different subspecies of *X. fastidiosa* (see pathogenicity tests) (Purcell et al., 1999; Purcell and Saunders, 1999; Monteiro et al., 2001; de Souza et al., 2003; Andreote et al., 2006; Saponari et al., 2014b). However, published back transmission experiments of CoDiRO strain from infected olive plants to healthy ones using *P. spumarius* were not successful (Saponari et al., 2014b). The authors reasoned that 'experiments with a larger sample size must be performed to prove or disprove *X. fastidiosa* transmission by *P. spumarius* to olives' (Saponari et al., 2014b). Transmission experiments presented in Saponari et al. (2014b) used field collected and CoDiRO strain infected *P. spumarius*. Inoculation was done with 8–10 spittlebugs/plant for an inoculation access period of 96 h at 26–28°C. Plants were tested by PCR for the presence of strain CoDiRO DNA at 30 day intervals. At the end of the experiment, two out of five periwinkle plants were positive, while all seven transmission tests conducted on olive plants failed.

The insect transmission experiments described in the submitted report were performed with grapevine and olive plants, while periwinkle plants were used as controls. Five field collected *P. spumarius* were placed in cages onto each plant for an inoculation access period of 48–96 h. These experiments were thus performed under conditions less stringent than those initially used to demonstrate the vector

status of *P. spumarius* and that had failed to result in successful back-transmission to olive. As tested by PCR, at least three out of the five insects used on each plant were positive for the CoDiRO strain. At the end of the experiment, approximately half the periwinkle plants and one-quarter of the olive plants tested were positive for CoDiRO by qPCR, while all grapevine plants (cv. Cabernet Sauvignon) subjected to infectious insects were negative.

Considering the evidence provided in the report, the Panel concluded that such experiments would require a larger number of test plants and higher infection pressures applied (higher number of insects/plant) in order to disprove the transmission of *X. fastidiosa* strain CoDiRO to grapevine by *P. spumarius*.

The Panel identified several elements of concern regarding the *P. spumarius* vector transmission experiments to infect grapevine with CoDiRO strain.

1. Relatively low rates of transmission of CoDiRO strain were observed with the two control plants, periwinkle (about 50%) and olive (26%). The report mentions other plant species on which the transmission experiments were also conducted (such as oleander, citrus, and peach GF 677), but the results are not presented. In total, small numbers of plants were exposed to infective *P. spumarius* (50 olive, 25 grapevine and 25 periwinkle plants). The Panel wonders if the imposed infection pressure was sufficient to guarantee a possibly rare transmission to grapevine.
2. The results of an earlier trial presented in Saponari et al., 2014b demonstrate that periwinkle is significantly more susceptible to the CoDiRO strain than olive and therefore periwinkle would not be an appropriate control plant for this type of test. An ideal control plant would have low but demonstrated susceptibility to the CoDiRO strain so that if these hypothetical control plants became infected and grapevine did not, it could be concluded that the experimental conditions were sufficient for rare inoculation events to occur.
3. The infective *P. spumarius* used for these transmission experiments were tested by qPCR and produced Cq values of 29–34. The Panel does not have sufficient information to translate these values directly into CFU/insect. However, these concentrations may be significantly lower than *X. fastidiosa* concentrations found in highly infective sharpshooters (Killiny et al., 2012). These low concentrations, in addition to the low transmission rates observed and the small number of plants tested, raise doubts about whether an inefficient transmission event to grapevine could have been observed.

As indicated above, vector transmission experiments require particular conditions and failures to transmit or to cause symptoms, even in compatible systems, are notorious (Hill and Purcell, 1995, 1997; Krugner et al., 2014; Saponari et al., 2014b).

Considering the evidence provided in the report, the vector transmission experiments do not provide sufficient evidence that the CoDiRO strain cannot be transmitted by insect vectors to grapevine plants. While all transmission experiments to grapevine were negative using *P. spumarius*, it cannot be excluded that the insects were not actively feeding, did not load sufficient bacteria into the xylem, or that other parameters for successful transmission were not met. Since *P. spumarius* only transmitted CoDiRO strain to a small number of the olive plants tested and because only a limited number of grapevine plants tested, the lack of transmission to *V. vinifera*, is not conclusive at this moment. The statement is even more valid for other *Vitis* species, not included in the trial.

3.5. Uncertainties

The uncertainties associated with field surveys conducted over a period of two years to detect infected grapevines are whether:

- the diagnostic tests performed (ELISA and PCR) would have sufficient sensitivity to detect *X. fastidiosa* strain CoDiRO at low concentrations in grapevine;
- sufficiently large numbers of grapevines per vineyard were tested even over repeated field visits. In general: whether the survey methodology was suitable (e.g., targeted vs. random sampling);

- all samplings were performed at periods suitable for the detection of *X. fastidiosa* in grapevine by the techniques used;
- there were sufficient infective vector populations (and therefore inoculum pressure) to infect grapevine with *X. fastidiosa* strain CoDiRO.

Pathogenicity tests were done following standard methods for experimental transmission of *Xylella* sp. to infect various host plants. Thus low uncertainty exists on the technical aspects of the experiment. However uncertainty exists whether:

- the number of plants included in the infectivity tests was sufficient, particularly considering the low effectiveness of artificial inoculations to infect olives, the susceptible host of the CoDiRO strain;
- the inoculation method used to infect grapevine was suitable, in particular whether a single inoculation at three points per plant would be sufficient to induce strain CoDiRO infections in grapevine;
- the bacterial DNA detected by qPCR one year after inoculation at the infection site was from viable bacterial cells that could present inoculum for vector transmission;
- other grapevine cultivars would respond similarly to cv Cabernet Sauvignon.

Despite the fact that the status of *P. spumarius* as a vector of CoDiRO strain is proven for periwinkle and olive, uncertainties of the vector transmission experiments are associated with:

- the ability of *P. spumarius* to transmit CoDiRO strain to grapevine plants;
- the number of insects used in the vector transmission trials, in particular whether the number of infective insects was sufficient to vector the pathogen to a plant that has a lower sensitivity to infection than olive, the major susceptible host;
- the number of the grapevine plants used, which is limited and would not allow detection of low frequency transmission events.

4. Conclusions

The Panel acknowledges the difficulty inherent in this complex pathogen/vector/host interaction and the challenge of providing evidence that grapevine is not susceptible to *X. fastidiosa* strain CoDiRO. The difficulties are best exemplified by the low efficiency to artificially infect even susceptible hosts such as olives. This is because many unknown factors (insect behaviour, environmental conditions etc.) are involved in the infection process which can make the experimental reproduction of observations/associations found in nature impossible. In addition, attempts to prove non-existing situations (i.e. grapevine is not a host) are associated with uncertainties on the correct experimental conditions used to (dis)prove a hitherto unknown situation.

Although CoDiRO strain has never been found in grapevines during the field surveys, due to the lack of data on vector presence in vineyards it cannot be concluded that inoculation from infective insect vectors occurred. Therefore, absence of disease symptoms and *X. fastidiosa* strain CoDiRO in field-grown grapevines do not prove that grapevine is a non-host for the CodiRO strain. Similarly, the unsuccessful transmission of CoDiRO by *P. spumarius* does not provide sufficient evidence that the behaviour of the insects on grapevine led to inoculations or that insects even fed on grapevine plants.

Following needle inoculations with bacteria isolated from olives, significant amounts of CoDiRO strain DNA were detected in grapevine plants by qPCR (Cq 24–25) as long as 1 year after inoculation. These concentrations were even higher than those measured in the infective *P. spumarius* used for the transmission experiments. The mere fact that bacterial DNA was only found localised at the points of inoculation does however not provide sufficient proof that the bacteria is not viable and could not potentially serve as a source of inoculum.

Results from surveys, pathogenicity tests and insect transmission trials are coherent and provide three converging lines of evidence that grapevine (*V. vinifera*) is not a major susceptible host of *X. fastidiosa* strain CoDiRO. However, from the experimental evidence, particularly the results of the

pathogenicity tests, it cannot be excluded that systemic infections occur and that even infections at limited foci could serve as a source of inoculum.

Therefore, based on the uncertainties of current data, the Panel considers it premature to conclude that *V. vinifera*, and because of the lack of data *Vitis* sp., cannot be infected and are not hosts of *X. fastidiosa* strain CoDiRO.

Documentation provided to EFSA

1. Scientific report 'Pathogenicity tests and analysis to verify the susceptibility of grapevines to *X. fastidiosa* strain CoDiRO'. September 2, 2015. Prepared by the National Research Council U.O.S. of Bari and the University of Bari Aldo Moro with the contribution of Saponari M and Boscia R. 30 p. Received as an annex to the mandate letter.

2. Annex 1 – Results of the field survey conducted on 1–9 September 2015. 11 p. Received as an annex to the mandate letter.

3. Annex 2 – Descriptive fiches of monitored vineyards. 72 p. Received as an annex to the mandate letter.

4. Annex 3 – Grapevine monitoring for XF: vineyard's selection criteria and sampling mode. 2 p. Received as an annex to the mandate letter.

5. Diagnostics on 174 samples – November-December 2013. Excel table. Received as email attachment on 16 October 2015.

6. Diagnostics on 2000 samples of certified mother plants and nursery stocks – December 2013-January 2014. Excel table. Received as email attachment on 16 October 2015.

7. Diagnostics on 135 samples – January 2015. 4 p. Received as email attachment on 16 October 2015.

8. Excel table with the total grapevines tested between November 2013 and November 2014. . Received as email attachment on 28 October 2015.

9. Results of transmission experiments carried out in 2014 on olive plants. Received as email attachment on 28 October 2015.

10. Details on the NGS sequencing on the De Donno xylem tissues. Received as email attachment on 5 November 2015.

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Abbreviations

CoDiRO	Complesso del Disseccamento Rapido dell'Olivo
Cq	quantification cycle value (qPCR)
CFU	colony-forming unit
EPPO	European and Mediterranean Plant Protection Organization
EU	European Union
MLST	Multilocus sequence typing
PCR	polymerase chain reaction
PLH Panel	EFSA Scientific Panel on Plant Health
VBNC	viable but non-culturable